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(RESEARCH ARTICLE)

Optimum γ- irradiation dose for the highest biological nitrogen fixation of bradyrhizobium

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Abstract

This study was conducted to evaluate the possibility of increasing Bradyrhizobium efficiency in fixing biological nitrogen in soil. Bradyrhizobium isolates were obtained from cowpea field in certain Iraqi provinces. Isolates were purified, propagated and authenticated. Isolates were then subjected to a range of Gamma ray dose of irradiation of 0 to 800 Gy. For all isolates number of nodules formed by all isolates increased with the increase of Irradiation dose to 400 Gy after which numbers of nodules of all isolates were markedly reduced. Highest nodules numbers of all isolates were observed under 400 GY irradiation dose. Under the same irradiation dose of 400 GY numbers of nodules differ with different isolates being the highest under isolate No. 2.

Keywords: N-15; Biological Nitrogen Fixation; Gamma Ray; Cowpea; Nodules; Cobalt -60

1. Introduction

Biological Nitrogen Fixation (BNF) is a natural process in legume crops, where atmospheric dinitrogen (N_2) is fixed into ammonia (NH_3) in plant root nodules by a symbiotic form of Rhizobia, a Gram-negative Proteobacteria. It depends on legume genotype as well as Rhizobia strain, but it also reflects the interaction between crop growth and plant-available N in the soil [1].

The expanded interest in ecology has drawn attention to the fact that BNF is ecologically safe and that it's greater exploitation can reduce the use of fossil fuels and can be helpful in reforestation and in restoration of misused lands to productivity [2], [3]. At present, BNF is of great practical importance because the use of nitrogenous fertilizers has resulted in unacceptable levels of water pollution (increasing concentrations of toxic nitrates in drinking water supplies) and the eutrophication of lakes and rivers [4], [5], [3]. While BNF may be almost sufficient to the needs of the organism, fertilizer is usually applied in a few large doses, up to 50% of which may be leached [3]. This wastes energy leads to serious pollution problems, particularly in water supplies.

Accordingly, there has been considerable investment in improving the capacity of legume plants for fixing atmospheric nitrogen [6], [7], [8]. This includes development of complex inoculant formulations designed to improve biological nitrogen fixation, and overall plant productivity [9].

Gamma Irradiation of legume plants as a host and/or Rhizobia were evaluated to improve the symbiosis through the effects on nodules referred to the first steps mechanism of nodule formation, as well as, inducing re-combinations in bacterial strains through conjugation to be selecting the efficient one in symbiosis to improve nodulation process.

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Gamma (γ) radiation has a narrow range of length high energy penetration power resulting from the nuclear disintegration of certain radioactive substances such as the isotopes Cobalt 60 (^{60} Co) and Cesium 137 (^{137}Cs).

Effects of radiation are accumulative [10]. Better N -fixing symbiosis may be brought by manipulating both rhizobia and plant hosts and by eventually creating an artificial rhizosphere. Numerous studies were conducted to induce genetic variations in Bradyrhizobium isolates to select the one of best symbiosis through alteration the expression of nodulin genes using gamma irradiation.

Therefore, this study was conducted to determine dose response curve of Gram-positive Bradyrhizobia isolates obtained from cowpea root in the field of Iraqi southern provinces. Gamma irradiation dose response curve of selected isolates will be utilized to determine the optimum Gamma Irradiation dose for the most efficient BNF of Bradyrhizobium isolate.

2. Methods

2.1. Collection of Nodules

Cowpea root nodules were collected from different field sites in governorates in Iraq, belonging to the most important cowpea production area in Iraq (Basrah, Mysan and Al Anbar).

Nodules were obtained and transferred into nodules collection vials as it was described by [11]. The vials were labeled and were stored at 4±1^{o}C for short-term storage.

2.2. Bacterial Isolates

Nodules were surface sterilized in series of surfactants in laminar air flow cabinet by immersing in 95% v/v ethanol for 10 seconds. Followed by 30% v/v solution of sodium hypochlorite for 4 min and then rinsed with sterile water. After that, they were placed in 3% v/v hydrogen peroxide followed by 5 times rinse in sterile water. The sterilized nodules were crashed with sterilized glass rod putting 1 ml sterilized distilled water in petri-dish. Furthermore, the nodule suspension was streak inoculated on yeast mannitol agar (YMA) [12] and incubated at 28 ^{o}C. Single colonies were selected and streaked onto YMA slant and kept at 4±1 ^{o}C for short term storage with sub culturing every 4 month. Long term storage was made by storing the culture broth in 10% glycerol at –20 ^{o}C [13].

2.3. Culture of Cowpea Rhizobium on YMA-CR Media

Different native cowpea rhizobium isolates were obtained from the cowpea root nodules collected from the respective sites were cultured by streak on YMA-CR medium under aseptic conditions. All the plates were labeled and incubated at 28 ^{o}C in inverted position and left for bacterial growth for 7 to 10 days in incubator, morphology of colony, nature of bacterial colony with Congo Red and shape and size of bacteria were observed for all isolates.

2.4. Authentication of Isolates by Infection Tests

Isolates obtained from the cowpea root nodules were authenticated as the cowpea rhizobium, the microsymbiont of cowpea. That is by examining their ability to infect cowpea roots to stimulate the root nodulation. Infection test was performed in sterile sand soil [14] and [15]. Sand was autoclaved for 3 times at 15 lbs (121 ^{o}C) for 20 minutes. Sterilized seeds of cowpea local cultivars were inoculated with 8 days old liquid of different bacterial isolates inocula prepared on Yeast Extract Mannitol Broth (YMB). The inoculated and uninoculated cowpea plants were grown for 4-5 weeks and examined the nodulation on their roots in both inoculated and uninoculated plants.

2.5. Yeast Manitol Agar-Bromothymol Blue (YMA-BTB) Reaction

All authenticated cowpea rhizobia cells, single bacterial colonies from the pure culture of all isolates were streaked on the plates containing YMA with 2.5 ml per liter of 1.0% alcoholic bromothymol blue (YMA-BTB). Then, the plates were labeled and incubated at 28 ^{o}C. The authenticity of each isolate was detected during 10 days incubation period, with observation for color of the dye indicator on the plates [12]. Fast growing rhizobia changed color of dye indicator to yellow while slow growing rhizobia turned the dye indicator to blue.

2.5.1. Preparation of Liquid Cowpea Rhizobium Inocula.

Cowpea Rhizobium isolates was maintained in liquid medium i.e. Yeast Extract Mannitol Broth (YMB) for inoculation on cowpea. YMB was prepared according to Vincent [16]. Five ml of YMB was dispensed on conical flasks and sealed

with aluminum foil and autoclaved. The autoclaved YMB flasks were then inoculated with cowpea rhizobium isolates, maintaining two replicates for each isolate. The cowpea rhizobium colonies from the YMA slants were transferred to the YMB flasks by sterilized inoculation lop. All activities were performed in laminar air flow cabinet. The inoculated YMB flasks were then incubated at 28 °C for 7 days in horizontal shaker with 120 rpm of rotation frequency.

2.6. Gamma Irradiation of Cowpea Rhizobium Isolates

This part of the work has been done in March 2015, at the laboratories of Soil and fertility department-IAEA / Vienna, Austria in Siebersdorf.

The wild type cowpea rhizobium isolate was inoculated into 40 ml YMB until turbidity (OD600) reached 0.15 approximately (10^7 CFUs/ml). The bacterial suspensions were then irradiated at 0, 50, 200, 400 and 800 Gy respectively. Irradiation was done at IAEA laboratories (Seibersdorf), using a cobalt -60 source (Dose rate 2 Gy/Sec.).

2.7. Mutant/s Efficiency Evaluation

Soil sand mixture was Autoclaved for 30 minutes at 121°C. Five seeds, previously one hour soaked in water, of cowpea were planted in each pot. 100 mL of specified inoculant were added per pot. Each inoculant was replicated 3 times in randomized complete block design.

After emergence plants were thinned to just 2 plants per pot. Phosphorus in 10 mg per Kg soil sand mixture was added to each pot. Plants were grown for six weeks in a green house at 20±1 °C day temperature and 16±1 °C for 10 hours night.

2.8. Preparation of Bacterial Cell Suspension

Suspension was prepared using Yeast Extract Mannitol Broth (YMB). Five hundred milliliters of this broth were prepared and autoclaved for 20 minutes at 121 °C. Colonies from each irradiated isolates with (0, 50, 200, 400 and 800 Gy) were incubated on Agar for 24 hr. of these inoculants sufficient amount were transferred to Yeast Extract Mannitol Broth (YMB) and incubated for 5 days in shaker incubator at 28 °C until growth reached the log phase at which turbidity at OD600 was 0.451. At this turbidity level there was approximately $6.0x10^8$ CFUs/ml.

2.9. Plant Harvesting

Plant root of each treatment were obtained by removing soil particle by light stream of water. Water on Plant roots was removed by soft tissue and number of nodules was calculated.

3. Results and Discussion

3.1. Authentication Test

The cowpea rhizobium bacterial isolates isolated from 3 Governorates in Iraq, were tested to identify their nodulation ability. Isolated bacteria were authenticated in nodulating cowpea, their original host plant, confirming their symbiotic status.

3.2. Bromothymol Blue Reaction

Bromothymol blue technique is used in bacteriology as a pH indicator in the agar, it changes to yellow in case of acid production or changes to deep blue in case of alkalization. This colorant is essentially used in order to differentiate Rhizobia, especially Bradyrhizobium which is Alkaline in reaction compared to Rhizobium isolates which is Acidic reaction [17].

The study showed colonies reaction of all the cowpea rhizobia on bromothymol blue (BTB) agar plates are slow growing group (Bradyrhizobium spp.).

3.2.1. Colony Characteristics and Growth Response on YMA-CR Medium

The bacterial colonies grown in YMA-CR plates were creamy white to translucent watery, smooth, circular and rose. The bacterial colonies did not absorb red color of Congo red when cultured in dark, which is the characteristic feature of bradyrhizobial colonies [18].

3.3. Soils of Siebersdorf

Soils of Siebersdorf table (1) used in this study is sandy clay loam soil with organic matter content in the range 5-5.2%. Total nitrogen content is 2.27% with 24.80 $mgKg^{-1}$ soil available P. According to these characteristics the soil is of good fertility level [19]. Adequate N and high plant available P will make this soil is appropriate for BNF process. The soil is also rich in Cations content due to its high CEC.

Table 1 Characteristics of soil from Siebersdorf/ Austria used in the study

Properties		Unit	Soil texture
Separations	Clay	$gmKg^{-1}$	333
	Silt		151
	Coarse silt		106
	Fine sand		179
	Coarse sand		231
pH 1:1			7.95
Total N		%	2.27
Total P		ppm	1115.00
Organic matter		%	5.52
Organic Carbon		%	3.21
C/N ratio			14.14
CEC		$Meq100^{-1}$	25.38
SAR			
Ion exchange	Ca	$Meq\ 100^{-1}$	22.03
	Mg		2.78
	Na		0.03
	K		0.22
	Mn		0.01
S(Ca,Mg, K, Na)			25.06
CEC			25.38
S/CEC		%	98.74

3.4. Effect of Gamma Irradiation on Cowpea Isolates

Most efficient isolates of Bradyrhizobium subjected to a range of γ-irradiation for inducing genetic variability. Isolates were initially grown in broth then subjected to assigned dose of γ-radiation. Irradiated isolates were transferred to YMA media for propagation. Cowpea seeds were inoculated with each irradiated isolate.

3.5. Effect of γ radiation on Number of Nodules

Response of three isolates of Bradirhizobium to given dose range of Gamma Irradiation of 0 to 800 Gy was given in Figures 1 to 3. Isolates response was measured by the number of nodules found on plant root.

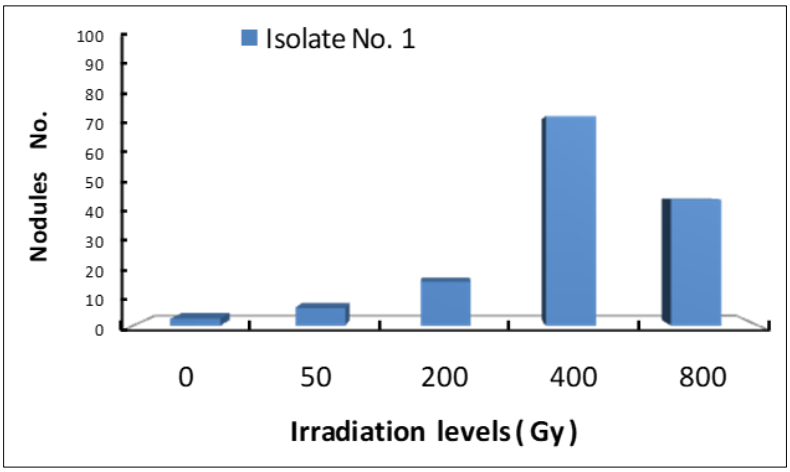

Figure 1 Response of Bradyrhizobium isolate 1 to a range of γ ray dose

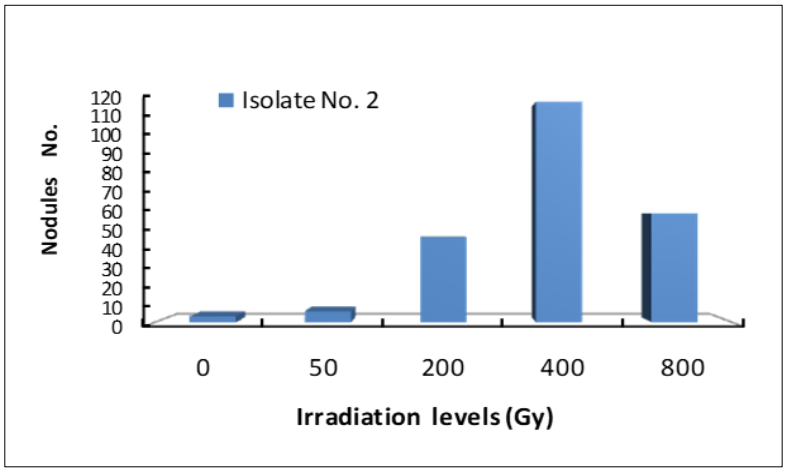

Figure 2 Response of Bradyrhizobium isolate 2 to a range of γ ray dose

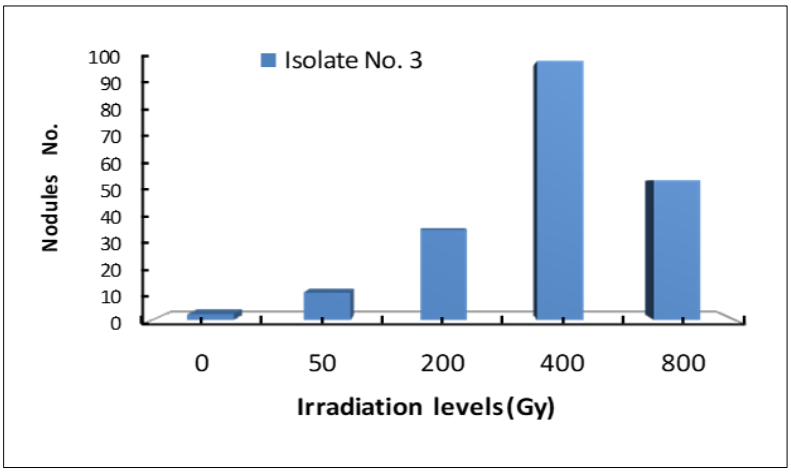

Figure 3 Response of Bradyrhizobium isolate 3 to a range of γ ray dose

The data clearly showed that number of nodules formed by each isolate was increased with the increase of gamma radiation dose to 400 Gy. Accordingly, Gamma dose of 400 Gy is the optimum level of Irradiation dose for the most efficient nodulation of the three isolates. At 800 Gy dose, number of nodules were markedly reduced under all three isolates which may indicate that this dose of Gamma irradiation is high enough to cause serious damage to isolate cells. These results are in agreement with those of [20], [21], [22] who had reported that Gamma Irradiation.

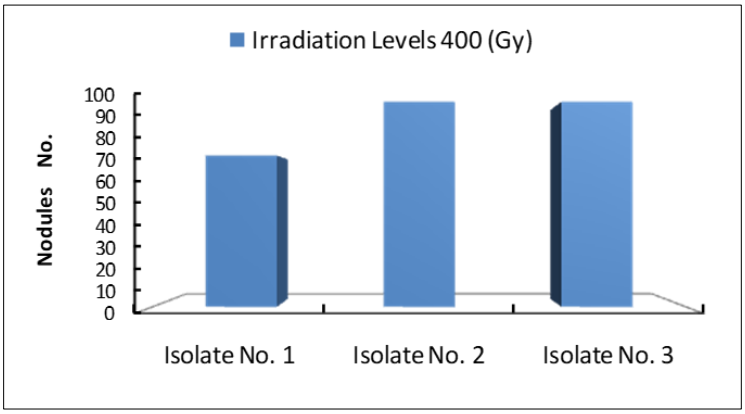

Figure 4 Number of Cowpea root nodules formed by 3 Bradyrhizobium isolate irradiated with 400 Gy γ radiation

Figure 4 shows number of colonies formed by each isolates subjected to 400 Gy dose of Gamma ray. The results clearly showed that number of nodules at the same radiation dose differ with different Isolates. Obviously, Isolate no. 2 showed the highest number of nodules which may indicate that this isolate under the given experimental condition is the most efficient isolate. Accordingly one may conclude that isolates nodulation efficiency increase with the increase of radiation dose to certain limit after which nodules number will decline. Furthermore, at same irradiation dose numbers of nodules differ with different isolates.

4. Conclusion

Methodology of using gamma irradiation technique to obtain new bacterial mutant of higher efficiency in fixing atmospheric Nitrogen was established. Gamma ray dose of 400 Gy was found to be the optimum irradiation dose for the highest possible BNF efficiency of Bradirhizobium.

Compliance with ethical standards

Acknowledgments

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Disclosure of conflict of interest

There is no conflict of interests to declare.

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